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**13. ABSTRACT** ***(Maximum 200 Words)***

Inactivation of tumor suppressor genes plays a causal role in the development of human cancers. The Deleted in Colorectal Cancer (DCC) gene is a putative tumor suppressor gene. Loss of heterozygosity at the DCC locus and loss of DCC expression has been observed in prostate cancer. Our previous data indicate that DCC expression induces apoptosis and cell cycle arrest of prostate tumor cells. By yeast two-hybrid screening, we have identified molecules that interact with the DCC cytoplasmic domain (dubbed DCC interacting proteins or DIPs). We hypothesize that the apoptotic signal of DCC is mediated by the DIPs. During this funding period, we have identified a novel molecule named DIP13, cloned its full length cDNA sequence, determined its exon-intron structure, mapped its interacting domain with DCC, demonstrated that DIP13 expression induces apoptosis and obtained evidence showing that DIP13 interacts with AKT, a key molecule for cell survival. Our results suggest that the DCC apoptotic signal is mediated by DIP13 that interferes with AKT cell survival pathway, resulting in cell death. Finally, we have cloned DIP13 beta, suggesting that DIP13 represents a family of molecules with at least two members.

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# Table of Contents

# Introduction

Tumor formation arises as a consequence of alterations in the control of cell proliferation and disorders in the interaction between cells and their surroundings. Uncontrolled cell proliferation is generally considered the result of oncogene activation and tumor suppressor gene inactivation, whereas alterations in cell-cell and cell-environment interactions are due in part to the malfunction of cell adhesion molecules. Interestingly the putative tumor suppressor gene DCC, encoding a cell adhesion molecule, may play a dual role.

Previously, we studied the DCC gene and its mRNA expression in human and rat prostatic carcinoma cells as well as in prostatic carcinoma tissues by RT-PCR and PCR-LOH (Gao et al, 1993). The DCC gene was present and expressed in normal prostatic cells. However, its expression was decreased or undetectable in all prostatic carcinoma cell lines either from human [4 cell lines] or rat [5 cell lines]. In patients, 12 out of 14 cases [86%] showed reduced DCC expression and 5 out of 11 informative cases [45%] showed loss of heterozygosity at the DCC locus. These results demonstrate that loss of DCC expression and loss of heterozygosity at the DCC locus are frequent features of prostatic carcinoma.

Subsequently, we found that DCC expression induces apoptosis or cell cycle arrest of human prostate tumor cells. Human DCC was expressed in DU145 and PC-3 carcinoma cells. Expression of DCC rapidly induced apoptosis of DU145 but not of PC-3 cells (Chen et al., 1999 and appendix 1). DNA fragmentation could be seen within 24 hours of DCC expression in DU145 cells. Apoptotic cells appeared starting at 18 hours of DCC expression and more than 90% of cells were apoptotic and floating at 48 hours. The morphological appearance of apoptotic cells coincided with the cleavage of poly (ADP-ribose) polymerase. Our results indicate that DCC-induced apoptosis is a rapid process, likely involving the activation of caspases. In PC-3 cells, DCC expression resulted in a flattened, enlarged morphology resembling G1 arrested cells. However, cell cycle distribution analysis by flow cytometry showed that cells were arrested at the G2/M phase. Furthermore, we found that DCC expression inhibited the Cdk1 kinase activity (Chen et al., 1999 and appendix 1).

In order to identify potential DCC signaling mediators, we used the DCC cytoplasmic domain as bait in yeast interaction trap screening. Thirteen DCC interacting proteins (DIPs) were identified. Four of the DIPs are novel genes.

Based on our preliminary studies, we propose a hypothetical signaling pathway(s) of DCC. An increasing level of DCC expression triggers DCC protein clustering. Depending upon cell genetic background, the cytoplasmic domain of the DCC protein then transduces signals resulting either in activation of caspases which induces apoptosis, or in inhibition of Cdk1 kinase activity which leads to G2/M cycle arrest.

The purpose of this study was to investigate the role of DCC interacting molecules (DIPs) in DCC signaling in human prostate carcinoma cells.

# Body

There were two aims in our proposal, i.e. (1) confirm the interaction of DCC with DIPs and (2) determine the necessity of DCC and DIP interaction in DCC-induced apoptosis and/or cell cycle arrest. We divided our work into two main tasks:

Task 1. Confirm the interaction of DCC with DIPs (month 1-15).

- Tagging of the DCC cytoplasmic domain, hsina and FKBP12 by PCR and sequencing to assure there is no mutation due to mis-incorporation.
- Transfection, immunoprecipitation and western analysis in prostate cells
- Generate N- and C-terminus truncated DCC cytoplasmic domain vectors
- Yeast two-hybrid assay using hsina, FKBP12 and the truncated DCC cytoplasmic domain.
- Confirm the interaction site(s) identified above in prostate cells

Task 2. Determine the necessity of DCC and DIP interaction in DCC-induced apoptosis and cell cycle arrest (month 16-30).

- Generate of DCC mutant(s) that lack hsina and/or FKBP12 interacting site.
- Generate, characterize, amplify and titrate recombinant DCC mutant adenovirus.
- Express wild type and mutant DCC in DU145 and PC-3 cells. Monitor apoptosis by light microscopy, DAPI staining, DNA laddering, flow cytometry and cleavage of PARP as well as cell cycle arrest by flow cytometry and Cdk1 kinase assay.
- Establish tetracycline-inducible hsina and FKBP12 expression cell lines.
- Monitor apoptosis by light microscopy, DAPI staining, DNA laddering, flow cytometry and cleavage of PARP as well as cell cycle arrest by flow cytometry and Cdk1 kinase assay.

During this funding period, we have performed most of the tasks outlined in our proposal. While our study progresses, we have also performed experiments that were not outlined but very relevant to our overall goal, i.e. signaling mechanism in DCC-induced apoptosis and/or cell cycle arrest in prostate tumor cells.

We published a paper in the journal of Oncogene (Chen et al., 1999, Appendix 1). In this paper, we demonstrated that expression of DCC activated caspase-3 and programmed cell death, or induced G2/M cell cycle arrest in tumor cells. In some cell lines, apoptosis was evident within 24 h of DCC expression. Timing of the appearance of apoptotic cells coincided with that of the cleavage of poly (ADP-ribose) polymerase, a substrate of caspase-3. In the G2/M cycle arrest cells, cdk1 activity was inhibited The DCC cytoplasmic domain was required for the induction of apoptosis and cycle arrest. Expression of the apoptosis inhibitory gene Bcl-2 was not able to abrogate the DCC-induced apoptosis.

Next, we mapped the interaction sites of all 13 DCC-interacting proteins on the DCC cytoplasmic domain. We found that DIP13 binds to a region between amino acid 1243 to 1264 (Fig. 1). This region

was shown to be required for inducing apoptosis (Mehlen et al., 1998). Therefore, we decided to concentrate our efforts on DIP13.

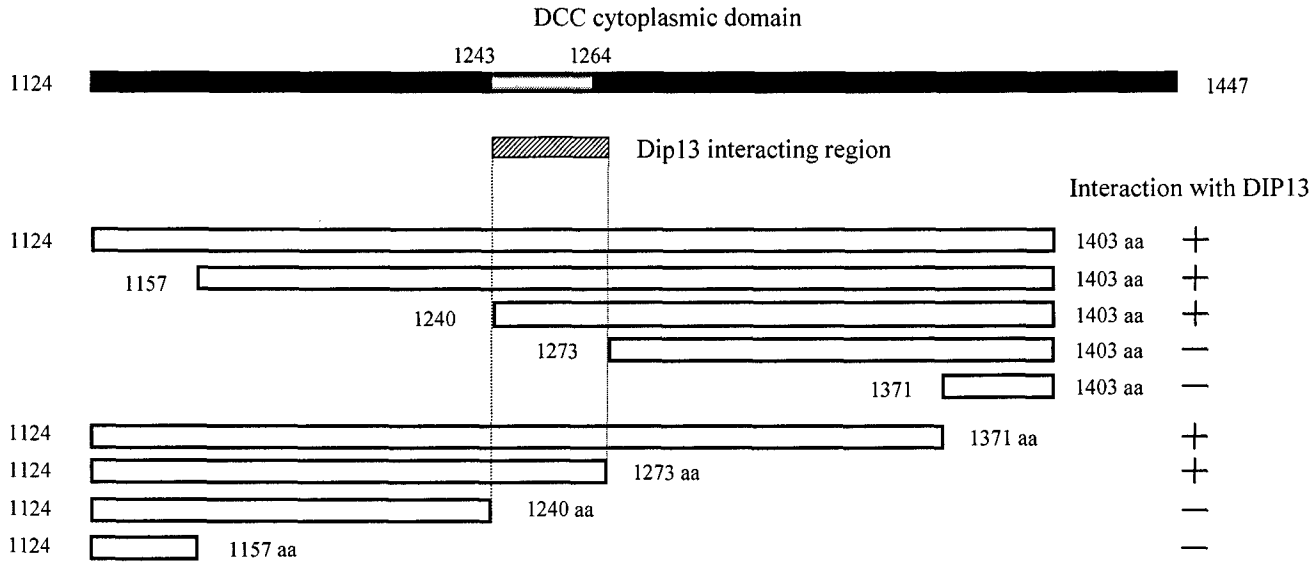


Fig. 1. Mapping of DIP13 interacting site on DCC

We cloned the full length DIP13 cDNA using a combination of cDNA library screening, 5'-RACE, PCR and sequencing. The human DIP13 mRNA has 3042 nucleotides with an open reading frame of 2130 bases (Fig. 2), encoding a protein of 709 amino acids.

cgcggggagctgtgggcggcagctgcgtctcctgccactgccctccctccgccacgATGCCGGGGATCGACAAGCT
GCCCATCGAGGAGACGCTGGAGGACAGCCCGCAGACAAGGTCTTTACTAGGTGTATTTGAAGAAGATGCYACAGCT
ATTTCCAACTATATGAACCAGTTGTATCAAGCTATGCATCGGATTTATGATGCACAGAATGAATTAAGTGCAGCAA
CACACCTGACCTCAAAACTTTTAAAAGAATATGAAAAACAGCGTTTTCCATTGGGAGGTGATGATGAAGTTATGAG
CTCTACATTGCAACAGTTTTCAAAAGTTATAGATGAGCTTAGCTCTTGTCATGCAGTGCTTTCAACTCAACTTGCT
GATGCCATGATGTTCCCCATTACCCAGTTTAAAGAAAGAGATCTGAAAGAAATACTAACATTAAAGGAAGTATTTC
AGATTGCAAGTAATGATCATGATGCTGCGATTAATAGATATAGCCGTTTATCAAAAAAAAGAGAAAATGACAAGGT
GAAGTATGAAGTAACAGAAGATGTGTACACATCCAGAAAGAAACAACACCAGACCATGATGCATTATTTTTGTGCA
TTAAATACTCTTCAGTACAAGAAGAAAATAGCATTGTTAGAACCTCTACTTGGGTACATGCAAGCTCAGATAAGTT
TCTTTAAGATGGGTTCTGAAAATCTTAATGAACAACTGGAAGAATTTTTAGCTAATATTGGAACAAGCGTTCAGAA
TGTTCGCAGGGAAATGGACAGTGATATAGAGACCATGCAACAGACAATAGAGGATTTGGAAGTAGCCAGTGATCCC
TTATATGTGCCTGACCCAGACCCCACCAAATTTCCTGTTAATCGAAATTTAACCCGAAAGGCTGGATACCTTAATG
CTAGGAATAAAACAGGCTTGGTGTCATCTACCTGGGACAGACAGTTTTACTTCACGCAGGGTGGAAATTTAATGAG
TCAGGCCCGTGGGGATGTAGCAGGAGGCCTGGCCATGGACATAGACAACTGTTCAGTGATGGCTGTGGACTGTGAA
GACAGACGATATTGTTTTCAGATCACCTCTTTCGATGGAAAAAAATCTTCAATTTTGCAAGCAGAGAGTAAAAAAG
ATCATGAAGAGTGGATCTGTACAATAAACAACATATCTAAACAAATATACTTAAGTGAAAATCCAGAGGAAACTGC
TGCACGAGTAAATCAATCAGCTCTGGAAGCTGTCACTCCTTCCCCATCTTTCCAGCAGAGGCACGAGAGCCTGCGG
CCAGCAGCAGGACAATCTCGGCCACCGACAGCTCGAACCAGCAGTTCAGGATCCTTAGGATCTGAGTCTACAAATT
TGGCTGCCCTCTCTCTAGATTCTCTTGTTGCCCCAGACACCCCAATACAGTTTGACATAATTTCTCCTGTGTGTGA
AGATCAGCCTGGCCAGGCAAAAGCCTTTGGCCAGGGAGGCAGGCGTACAAATCCATTTGGAGAATCTGGAGGAAGT
ACAAAATCTGAAACTGAAGATTCTATTCTTCATCAGTTATTTATTGTCCGATTCCTTGGTTCAATGGAGGTGAAAT
CAGATGACCATCCAGATGTTGTTTATGAAACTATGCGCCAAATCTTAGCTGCCCGGGCCATCCATAACATCTTTCG
TATGACAGAATCGCATTTATTAGTCACTTGTGACTGTTTAAAGTTAATTGATCCACAGACACAAGTTACAAGGCTC
ACGTTTCCATTACCTTGTGTAGTTTTGTATGCTACACACCAGGAAAATAAGCGCCTTTTTGGATTTGTTCTTCGGA
CATCAAGCGGGAGAAGTGAAAGTAATCTGTCATCAGTCTGCTATATATTTGAGTCAAACAATGAGGGGGAAAAGAT
ATGTGATTCTGTTGGACTGGCAAAACAGATAGCTTTGCATGCTGAACTGGATCGTAGGGCATCAGAAAAACAAAAA
GAAATAGAGAGAGTAAAAGAGAAGCAACAGAAAGAACTCAATAAACAAAAACAGATTGAAAAGGACTTGGAAGAAC
AAAGTCGGTTGATAGCTGCTTCCAGTAGACCAAACCAAGCCAGTAGTGAGGGGCAGTTTGTTGTCCTTAGCAGTAG
CCAGTCAGAAGAGAGTGATTTGGGAGAAGGAGGAAAGAAGAGAGAATCAGAAGCATAAgcttatacttttggtaaa
tattccccccttggaatttgacagtttctatggtgaaatggcagaaggtaacaactatgttgaaatatcaaggagga
gattaagctttatatttgcctaattgttgtagctacattttaaaaaaaagattgaacttgatgacttaagtttgca
ttgatctttttcccccttaaacataatgtactatgtattaacatcctaaagggaacctgctcatctccctgaagca
gactgctgaagaaatacatttgctcaagaatttttccgtcaaatttgtgaacttttaattcctgcaattgtaattg
gctgtgcccataggaatcatttattcttcaggcttagacattcatggatatgcttttctttcattaggaacatttt

gctggtgatgaggaagcatttagctgaataagtttaaagccctttataccggaattctacaagtttgcaaatattt
taacaatattaaatgtgcaatagaacttttttaaaataattggaacgggattttacagttaaggtttcaaattgtg
gcaggtggtactgttgatctcagggtactttctgggattgctcacatttctctaatgtactgcacttgatgccagt
cggaaagaagcttaagtgtcttcagttcaagattgatagagcccttggcattttattatcacattcttagttctca
ggttgggacttcaattactgctgcagagccgtagtggttaaaaataagatattggaatttattaaaagatttttgt
tcaatacattttagattaggattgacaagtaaagatactgctatggaatgtaaaaaaaaaaaaaaaaaaaaaaaa
aa

Fig. 2. DIP 13-cDNA sequence. Upper case: open reading frame.

We also determined the intron-exon structure of DIP13. The DIP13 gene consists of 22 exons and all intron-exon junctions confine to the gt-ag role (Fig. 3)

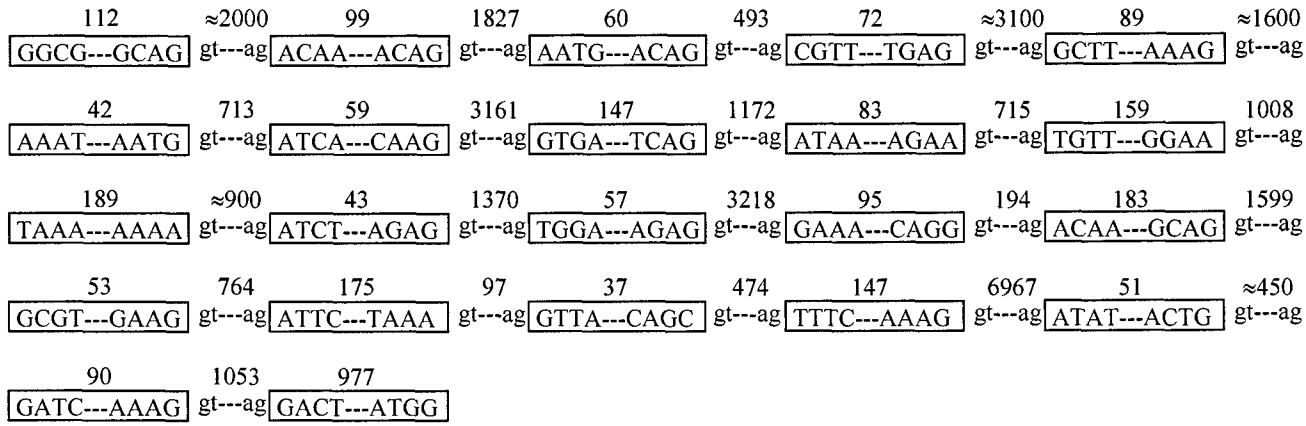


Fig. 3. Human DIP13 gene structure.

The DIP13 protein has a PH (pleckstrin homology) and PTB (Phosphotyrosine binding) domain (Fig. 4). The pleckstrin homology domain is an approximately 100-residue protein module that is present in many kinases, isoforms of phospholipase C, GTPases, GTPase-activating proteins and nucleotide-exchange factors. Many PH-domain-containing proteins interact with GTP-binding proteins. Phosphotyrosine binding (PTB) domains have been identified in a large number of proteins. This domain usually binds in a phosphotyrosine-dependent fashion to peptides that form a b turn. However, the PTB domains have also been found to participate in phosphotyrosine-independent interactions (Margolis et al., 1999). The ability

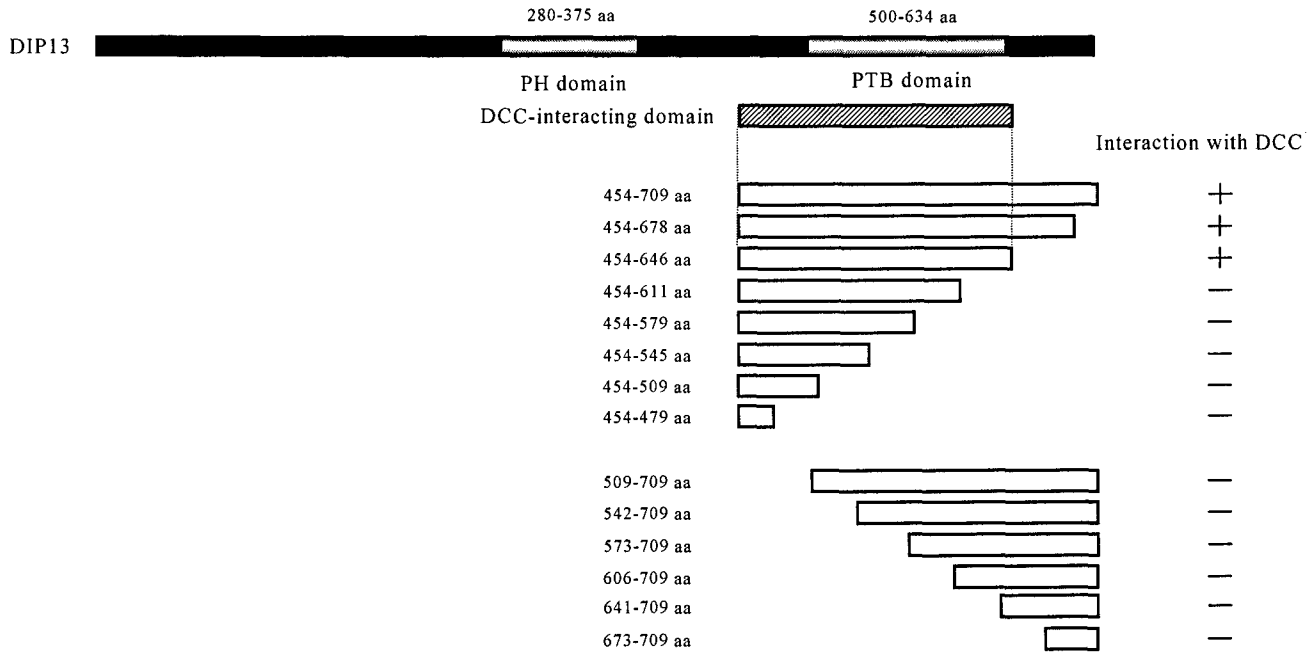


Fig. 4. Mapping of DCC interacting site on DIP13.

of the PTB domain to bind peptides in a phosphotyrosine-dependent and -independent fashion allows this domain to be involved in diverse cellular functions. We found that DIP13 interacts with DCC through its PTB domain (Fig. 4). Interestingly, Mitsuuchi et al. (1999) identified a gene dubbed APPL that interacts with AKT, a key molecule controlling cell survival and apoptosis. It turns out that APPL is identical to DIP13. Our preliminary results confirm DIP13-AKT interaction (data not shown).

DIP13 was expressed using a tetracycline-inducible system in tumor cells (Fig. 5). Forced expression of DIP13 induced apoptosis (Fig. 5).

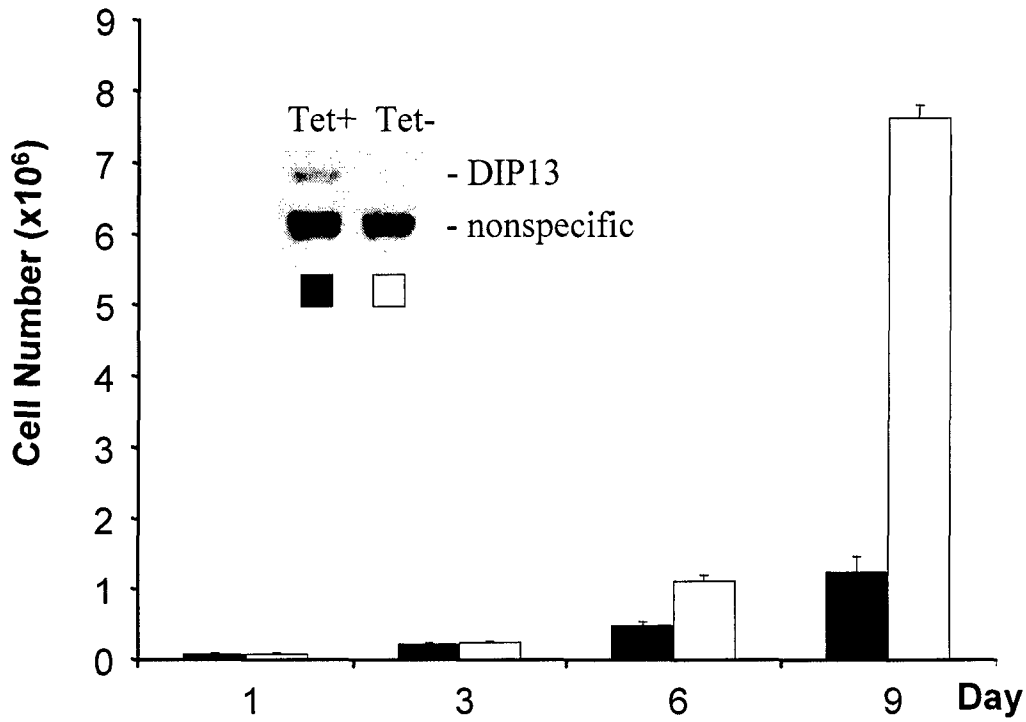


Fig. 5. Expression of DIP13 and cell survival.

Recently, we have identified a novel gene named DIP13 beta. We renamed the original DIP13 as DIP13 alpha. The beta protein is 52% identical and 69% homologous to the alpha protein. The highest homology lies within PH and PTB domains (Fig. 6)

```
Alpha  MPGIDKLPIEETLEDSPQTRSLLGVFEEDATAISNYMNQLYQAMHRIYDAQNELSAATHLTSKLLKEYEKQRFPLGGDDEVMSSTLQQFSKVIDELSSCH
Beta   --AV---LL--A-Q---------S------GTLTD-T---L---Q-V-G----MCL--QQL--Q-LA----N-A--KG--EVI---HY----V---NLL-

101    AVLSTQLADAMMFPITQFKERDLKEILTLKEVFQIASNDHDAAINRYSRLSKKRENDKVKYEVTEDVYTSRKKQHQTMMHYFCALNTLQYKKKIALLEPL
101    TE-AK----T-VL-----R----T-VS---DL------E--LSMAK----P--K--E---T--GKE-AAA-R---LSSLQ-Y----A---R-QMAMM--M

201    LGYMQAQISFFKMGSENLNEQLEEFLANIGTSVQNVRREMDSDIETMQQTIEDLEVASDPLYVPDPDPTKFPVNRNLTRKAGYLNARNKTGLVSSTWDRQ
201    I-FAHG--N---K-A-MFSKRMDS-LSSVADM-QSIQV-LEAEA-K-RVSQQE-LSVDESV-T--S-VAAPQI----IQ------L-------TT--E-L

301    FYFTQGGNLMSQARGDVAGGLAMDIDNCSVMAVDCEDRRYCFQITSFDGKKSSILQAESKKDHEEWICTINNISKQIYLSENPEETAARVNQSALEAVTP
301    YF--------C-P--A-----IQ-L--------------------TPN--SGI------R-EN----------R----TD---AV-IKL--T--Q----

401    SPSFQQRHESLRPAAGQSRPPTARTSSSGSLGSESTNLAALSLDSLVAPDTPIQFDIISPVCEDQPGQAKAFGQGGRRTNPFGESGGSTKSETEDSILHQ
401    IT--GKKQ--SC-SQNLKNSEMENENDKIVPKATASLPE-   EE-I--G-------VL-AT-     FLDQNR-S--------TEDESFP-A-----Q-

501    LFIVRFLGSMEVKSDDHPDVVYETMRQILAARAIHNIFRMTESHLLVTCDCLKLIDPQTQVTRLTFPLPCVVLYATHQENKRLFGFVLRTSSGRSESNLS
493    M---------A--TDSTTE-I--A---V----------------M--SQS-R--------S-ANFE-TS-TQF-A-------V---I-VPESTG-ES--

601    SVCYIFESNNEGEKICDSVGLAKQIALHAELDRRASEKQKEIERVKEKQQKELNKQKQIEKDLEEQSRLIAASSRPNQASSEGQFVVLSSSQSEESDLGE
593      T------S------YAIN-G-E-I                                        EVQ--P-ALAQ-MLSIPLTN     D-KY-L-NDQPDDDDGNPN

701    GGKKRESEA
656    EHRGA----
```

Fig. 6. Protein sequence comparison between human DIP13 alpha and beta. - denotes an identical residue, boxed letters indicate PH and PTB domains.

The identification of DIP13 beta suggests that DIP13 represent a novel family of signaling molecules with PH and PTB domain. Both DIP13 alpha and beta appear to be remarkably conserved between Homo Sapiens and Mus Musculus. Partial sequence of the mouse DIP13alpha indicates that 201 amino acids of the first 204 residues are identical between human and mouse alpha (Fig. 7).

```
Human MPGIDKLPIEETLEDSPQTRSLLGVFEEDATAISNYMNQLYQAMHRIYDAQNELSAATHLTSKLLKEYEKQRFPLGGDDEVMSSTLQQFSKVIDELSSCH
Mouse ----------------------------------------------------------------------------------------------------

      AVLSTQLADAMMFPITQFKERDLKEILTLKEVFQIASNDHDAAINRYSRLSKKRENDKVKYEVTEDVYTSRKKQHQTMMHYFCALNTLQYKKKIALLEPL
      AVL------------S-----------------------------------------------------C------------------E-----------

      LGYMQAQISFFKMGSENLNEQLEEFLANIGTSVQNVRREMDSDIETMQQTIEDLEVASDPLYVPDPDPTKFPVNRNLTRKAGYLNARNKTGLVSSTWDRQ
      ----

      FYFTQGGNLMSQARGDVAGGLAMDIDNCSVMAVDCEDRRYCFQITSFDGKKSSILQAESKKDHEEWICTINNISKQIYLSENPEETAARVNQSALEAVTP

      SPSFQQRHESLRPAAGQSRPPTARTSSSGSLGSESTNLAALSLDSLVAPDTPIQFDIISPVCEDQPGQAKAFGQGGRRTNPFGESGGSTKSETEDSILHQ

      LFIVRFLGSMEVKSDDHPDVVYETMRQILAARAIHNIFRMTESHLLVTCDCLKLIDPQTQVTRLTFPLPCVVLYATHQENKRLFGFVLRTSSGRSESNLS

      SVCYIFESNNEGEKICDSVGLAKQIALHAELDRRASEKQKEIERVKEKQQKELNKQKQIEKDLEEQSRLIAASSRPNQASSEGQFVVLSSSQSEESDLGE

      GGKKRESEA
```

Fig. 7. Alignment of human and mouse DIP13 alpha.

Comparison between human and mouse DIP13beta shows an overall 92% identity (Fig. 8).

```
Human MPAVDKLLLEEALQDSPQTRSLLSVFEEDAGTLTDYTNQLLQAMQRVYGAQNEMCLATQQLSKQLLAYEKQNFALGKGDEEVISTLHYFSKVVDELNLLH
Mouse ------------------A-------------------------------------------R----------------------------M----G--

      TELAKQLADTMVLPIIQFREKDLTEVSTLKDLFGLASNEHDLSMAKYSRLPKKKENEKVKTEVGKEVAAARRKQHLSSLQYYCALNALQYRKQMAMMEPM
      --------------V----------------------S--------------------A---IV--------------------------RA-----L

      IGFAHGQINFFKKGAEMFSKRMDSFLSSVADMVQSIQVELEAEAEKMRVSQQELLSVDESVYTPDSDVAAPQINRNLIQKAGYLNLRNKTGLVTTTWERL
      --X---------R-------S--G-----K--------------D------------S-------I---TA---------T--------------------

      YFFTQGGNLMCQPRGAVAGGLIQDLDNCSVMAVDCEDRRYCFQITTPNGKSGIILQAESRKENEEWICAINNISRQIYLTDNPEAVAIKLNQTALQAVTP
      ----------------------------------------------S--S--P----------Y------V------------------------------

      ITSFGKKQESSCPSQNLKNSEMENENDKIVPKATASLPEAEELIAPGTPIQFDIVLPATEFLDQNRGSRRTNPFGETEDESFPEAEDSLLQQMFIVRFLG
      ------------S---I---DI-D  -N--------I--T--------------------------G----------G--------------------

      SMAVKTDSTTEVIYEAMRQVLAARAIHNIFRMTESHLMVTSQSLRLIDPQTQVSRANFELTSVTQFAAHQENKRLVGFVIRVPESTGEESLSTYIFESNS
      ---------A-------------------------------T------------C-----------------------------------------------

      EGEKICYAINLGKEIIEVQKDPEALAQLMLSIPLTNDGKYVLLNDQPDDDDGNPNEHRGAESEA
      -------------------------R----V-------------A--TG-S-S-N-------
```

Fig. 8. Alignment of human and mouse DIP13 beta.

In summary, we have identified a novel molecule named DIP13alpha, cloned its full length cDNA sequence, determined its exon-intron structure, mapped its interacting domain with DCC, demonstrated that DIP13alpha expression induces apoptosis and obtained evidence showing that DIP13 interacts with AKT, a key molecule for cell survival. Our results suggest that the DCC apoptotic signal is mediated by DIP13 that interferes with AKT cell survival pathway, resulting in cell death. Finally, we have cloned DIP13 beta, suggesting that DIP13 represents a family of molecules with at least two members.

## Key research accomplishments

- Mapping DIP13 alpha interacting site on DCC
- Mapping DCC interacting site on DIP13 alpha
- Cloning full-length cDNA of human DIP13 alpha and determining its gene structure.
- Showing expression of DIP13 alpha induces apoptosis
- Cloning human DIP13 beta
- Partial cloning mouse DIP13 alpha
- Cloning mouse DIP13 beta

## Reportable outcome

- One manuscript (Appendix 1)
- One abstract (Appendix 2)
- Polyclonal anti-human DIP13 alpha
- DIP13alpha-inducible expression cell line
- Human DIP13alpha cDNA plasmid
- Human DIP13beta cDNA plasmid
- Mouse DIP13beta cDNA plasmid

## Conclusions

Previously, we observed loss of heterozygosity at the DCC locus and loss of DCC expression in both human prostate tumor tissues and prostate carcinoma cell lines, indicating that inactivation of the DCC gene may contribute to prostate cancer development. We now show that forced expression of DCC induces G2/M cell cycle arrest and apoptosis of prostate carcinoma cells. By yeast two-hybrid screening, we have identified several molecules that interact with the DCC cytoplasmic domain. We proposed to study the signaling mechanism in DCC-induced apoptosis and/or cell cycle arrest in prostate tumor cells. Several important outcomes have been resulted from our studies:

First, our results suggest that DIP13alpha is a potential mediator of the DCC apoptotic signal. Should loss of DCC function be confirmed to play a role in prostate cancer development, inactivation of DCC may serve as a prognostic/diagnostic marker for prostate cancer progression.

Secondly, since DCC or DIP13 alpha expression induces apoptosis of prostate tumor cells, the DCC-DIP13 pathway may be exploited for prostate cancer gene therapy.

Thirdly, we have identified a novel family of signaling molecules. The fact that these molecules contain PH and PTB domain indicates they may interact with many other molecules. Members of the DIP13 family may play critical roles in growth factor signaling and apoptosis.

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# Appendix

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# Induction of apoptosis and G2/M cell cycle arrest by DCC

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**The Deleted in Colorectal Cancer gene (DCC) encodes a cell surface receptor that belongs to the Ig superfamily. Inactivation of the DCC gene has been implicated in human tumor progression. However, little is known about the biological function of the DCC protein. In the present study, we demonstrated that expression of DCC activated caspase-3 and programmed cell death, or induced G2/M cell cycle arrest in tumor cells. In some cell lines, apoptosis was evident within 24 h of DCC expression. Timing of the appearance of apoptotic cells coincided with that of the cleavage of poly (ADP-ribose) polymerase, a substrate of caspase-3. Expression of the apoptosis inhibitory gene Bcl-2 was not able to abrogate the DCC-induced apoptosis. In the G2/M cycle arrest cells, cdk1 activity was inhibited. Our results suggest that the DCC protein may transduce signals resulting in activation of caspases or inhibition of Cdk1. These data provide a possible mechanism by which DCC suppresses tumorigenesis.**

**Keywords:** DCC; apoptosis; caspase; tumor suppressor gene; cell cycle; Cdk1

## Introduction

Deleted in Colorectal Cancer (DCC) was originally identified by virtue of its high frequency of deletion in colon carcinoma. The DCC gene was mapped to chromosome 18q21.2 and isolated by positional cloning (Fearon *et al.*, 1990; Cho *et al.*, 1994). Deduced amino acid sequence shows a high homology with the neural cell adhesion molecule (N-CAM) and other related cell surface glycoproteins of the Ig superfamily (Fearon *et al.*, 1990; Cho *et al.*, 1994). Frequent loss of heterozygosity at the DCC locus and loss of DCC expression have been observed in human colon (Fearon *et al.*, 1990; Thiagalingam *et al.*, 1996) and prostate carcinomas (Gao *et al.*, 1993) as well as in pancreatic, gastric, esophageal, bladder, breast, head and neck carcinomas and male germ cell tumors, neuroblastoma, glioma, hematologic malignancy (Fearon, 1996, references therein). These results suggest that inactivation of the DCC gene may be involved in the development of human cancers. However, the biological function of DCC is still largely unknown.

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Recently, the tumor suppressor gene Deleted in Pancreatic Cancer (DPC4 or smad4) has been cloned and shown to play an important role in TGF-$\beta$ signaling. The DPC4 (smad4) gene is located in close proximity to the DCC locus on chromosome 18q. Because of this close proximity and the uncertainty of DCC function, there was a question whether DPC4 rather than DCC is the tumor suppressor gene that is inactivated in colon carcinoma cells. In order to address this question, the loss of heterozygosity at 18q has been re-evaluated in colon tumor samples (Thiagalingam *et al.*, 1996). The results suggest that the DCC, but not the DPC4, is the most frequently altered gene on chromosome 18q13.3-21.3. In this study, we found that forced DCC expression induces apoptosis or cell cycle arrest, providing a potential mechanism of DCC action.

## Results and discussion

In order to determine the function of DCC, human DCC cDNA was expressed in colon DLD-1 and prostate DU145, PC-3 carcinoma cells by recombinant adenovirus infection. Mock and control adenovirus-infected DLD-1, DU145 and PC-3 cells did not express a detectable level of DCC, whereas the 180 kD DCC protein was easily detected in DCC adenovirus-infected cells (Figure 1a). Expression of DCC rapidly induced apoptosis of DLD-1 and DU145 but not PC-3 cells. DNA fragmentation could be seen within 24 h of DCC expression in DLD-1 and DU145 cells (Figure 1b). DNA fragmentation was also evident by flow cytometry analysis of DNA content. At 48 h postinfection, mock and control adenovirus-infected DU145 cells had a similar G1, S and G2/M phase distribution, whereas DCC adenovirus-infected DU145 cells had a large population of cells with DNA contents smaller than G1 phase cells (Figure 1c), a characteristic of apoptotic cells. DCC protein expression in DU145 cells was detectable at 12 h and reached a plateau approximately 30 h postinfection (Figure 2a). Apoptotic cells could be seen starting at 18 h postinfection and more than 90% of cells were apoptotic and floated at 48 h postinfection (Figure 2b). The morphological appearance of apoptotic cells coincided with the cleavage of poly (ADP-ribose) polymerase (Figure 2c). It has been demonstrated that proteolytic cleavage of PARP by caspases is a widespread phenomenon in cells undergoing apoptosis (Kaufman *et al.*, 1993; Lazebnik *et al.*, 1994; Nicholson *et al.*, 1995; Tewari *et al.*, 1995). Similar kinetics of DCC-induced programmed cell death was observed in DLD-1 cells (data not shown).

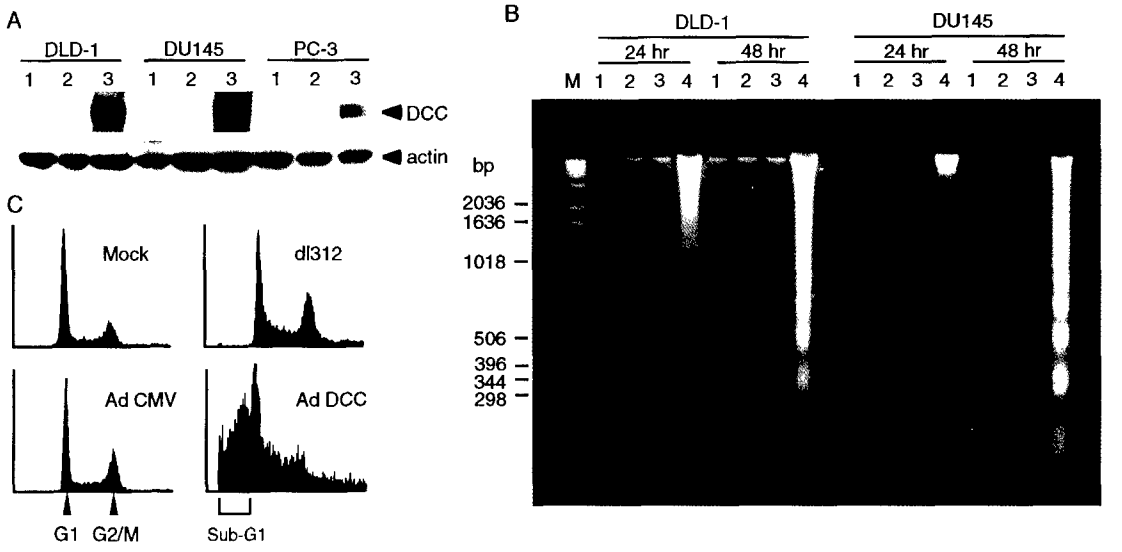


**Figure 1** Induction of apoptosis by DCC. (**a**) Cells were mock-infected (lane 1) and infected at MOI 5 with a control adenovirus dl312 (lane 2) or AdDCC adenovirus (lane 3). Cells were collected 24 h postinfection, lysed and used for Western detection of DCC. Membrane was stripped and reprobed for $\beta$-actin to show protein loading. (**b**) Cells ($1 \times 10^6$) were mock-infected (lane 1), and infected at MOI 5 with control adenovirus AdCMV (lane 2), dl312 (lane 3) or AdDCC (lane 4). Samples were collected at 24 and 48 h postinfection and used for DNA fragmentation assay. (**c**) DU145 cells ($2 \times 10^6$) were mock infected or infected at MOI 5 with control adenovirus AdCMV, dl312 and AdDCC. Cells were collected at 48 h postinfection, fixed and analysed for cell cycle distribution

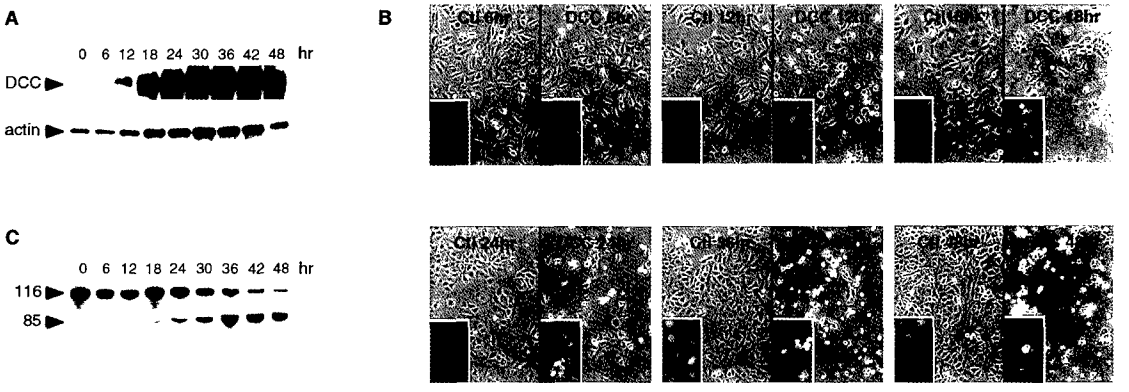


**Figure 2** Kinetics of DCC-induced apoptosis in DU145 cells. Cells ($1 \times 10^6$) were seeded in 100-mm culture dish each containing a coverslip. Next day, cells were mock-infected or infected with dl312 and AdDCC at MOI 5 in 2 ml culture medium for 1 h at 37°C with shaking every 10 min. Three ml medium were added after infection. Cells were collected at 6 h intervals for a period of 48 h for Western detection of DCC and $\beta$-actin protein (**a**), at which point cell morphology was monitored by light microscopy (**b**; phase contrast photos). Cells attached to the coverslip were fixed and used for DAPI staining (**b**; fluorescent photos). Cell samples collected in the experiment A were also used for the analysis of PARP cleavage. The 116 kD PARP protein and the 85 kD apoptosis-related cleavage fragment were detected by Western blotting (**c**)

Our results indicate that the DCC-induced apoptosis is a rapid process, likely involving the activation of caspases. A loss of DCC expression is a common feature in many cancer cells. However, the levels of DCC mRNA and protein are usually very low in normal tissues, to the point that they are hardly detectable by Northern and Western blotting. Immunostaining of human colon tissue indicates that DCC is expressed only in the fully differentiated epithelial secretory cells, the goblet cells, and in late-stage adenomas and in nonmucinous carcinoma, DCC expression is lost (Hedrick *et al.*, 1992). In order to compare the level of DCC expression in our experimental system to that *in vivo*, human colon carcinoma DLD-1 cells were infected with AdDCC or d1312. The cells were collected at 18 h post-infection. Cell samples as well as human intestinal tissue sections were simultaneously used for immunostaining of the DCC protein. The secretory cells of intestinal tissue and AdDCC-infected cell culture gave strong DCC staining (Figure 3a). The intensity of DCC protein staining was quantified by optical densitometry (Figure 3b). The mean value for the negative control sample was 64, for secretory cells 294 and for AdDCC-infected DLD-1 cells 259. These results indicate that, under our experimental condition, adenoviral mediated expression of DCC in culture results in a level of the DCC protein, which is comparable to that in human

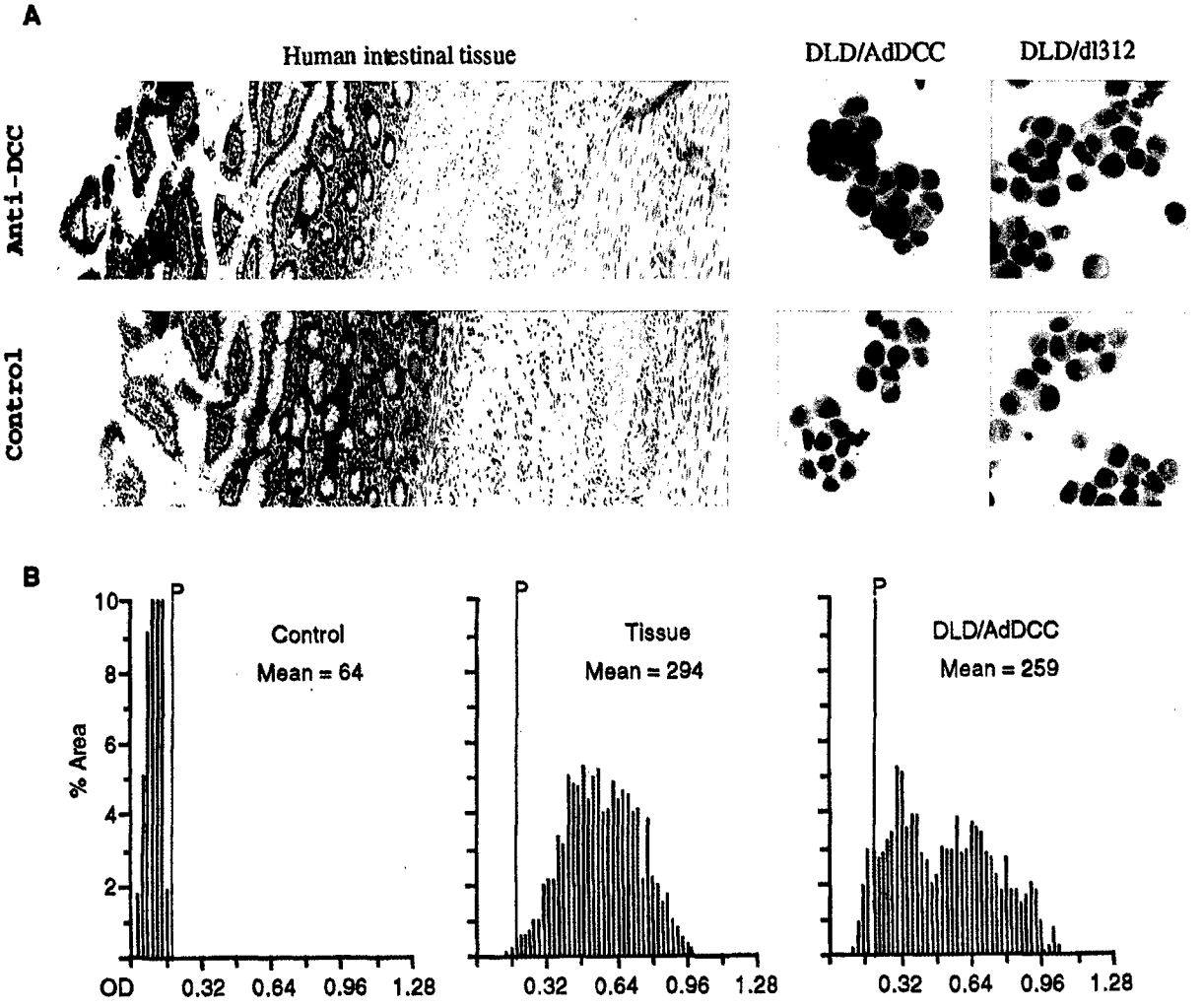


**Figure 3** Comparison of DCC protein level *in vitro* and *in vivo*. (**a**) Human intestinal tissue sections were incubated with anti-DCC cytoplasmic domain monoclonal antibody (upper left panel) or normal mouse IgG (lower left panel), followed by anti-mouse IgG conjugated with peroxidase antibody. DLD cells were infected with AdDCC (middle panels) or d1312 (right panels) at MOI 5. Cells were collected 18 h post-infection and incubated with anti-DCC cytoplasmic domain monoclonal antibody (upper middle and upper right panels) or normal mouse IgG (lower middle and lower right panels), followed by anti-mouse IgG conjugated with peroxidase antibody. Note that DCC expression (red color) is restricted to secretory cells in mucosa. (**b**) The intensity of DCC protein staining was determined by the Becton Dickinson CAS 200 Cell Analysis System. The cytoplasmic area of 100 individual cells from each sample was measured and the total pixel histograms for control, human tissue and DLD-1 cells infected with AdDCC (moi 5) were shown. OD: optical density. P: OD higher than the *P* value was considered positive

intestinal secretory cells. It is well known that the fully differentiated epithelial cells of the GI tract have a rapid turnover rate, i.e. cells die within 2–3 days. Therefore, it is possible that DCC is only expressed to a high level during the differentiation of basal epithelial cells to secretory cells, resulting in rapid cell death. Thus DCC may function as an apoptosis inducer in human tissue.

It is known that some members of the Bcl-2 gene family can inhibit or delay apoptosis stimulated by a variety of signals (White, 1996; Oltvai and Korsmeyer, 1994; Boise *et al*., 1993). To determine whether expression of Bcl-2 or Bcl-xL can inhibit DCC-induced apoptosis, SHEP-1 cell, an epitheloid subclone of the human neuroblastoma line SK-N-SH, and SHEP-1 clones expressing Bcl-2 or Bcl-xL were infected with control adenovirus, DCC adenovirus or mock-infected. Programmed cell death was determined at different time points by cell morphology and DNA fragmentation assay. Forced DCC expression induced apoptosis of SHEP-1 cell and clones expressing Bcl-2 or Bcl-xL (data not shown). This result suggests the possibility that Bcl-2 or Bcl-xL may not be able to block DCC-induced programmed cell death. To confirm this observation, DU145 and DLD-1 cells were transfected with a Bcl-2 expression vector (pcDNA Bcl-2-flag) and control vector (pcDNA3). Pooled populations as well as individual colonies of Neo and Bcl-2 were obtained. No Bcl-2-flag epitope were detected in pooled Neo populations of DU145 and DLD-1 cells (Figure 4a). However, the Bcl-2-flag protein was easily detectable in pooled Bcl-2 populations and individual colonies of DU145 and DLD-1 cells (Figure 4a). No endogenous Bcl-2 protein was detected in DU145 cells (Figure 4a), a result similar to that described by another group (Haldar *et al*., 1996). Endogenous Bcl-2 protein, at a lower molecular weight than that of the transfected Bcl-2-flag protein, was seen

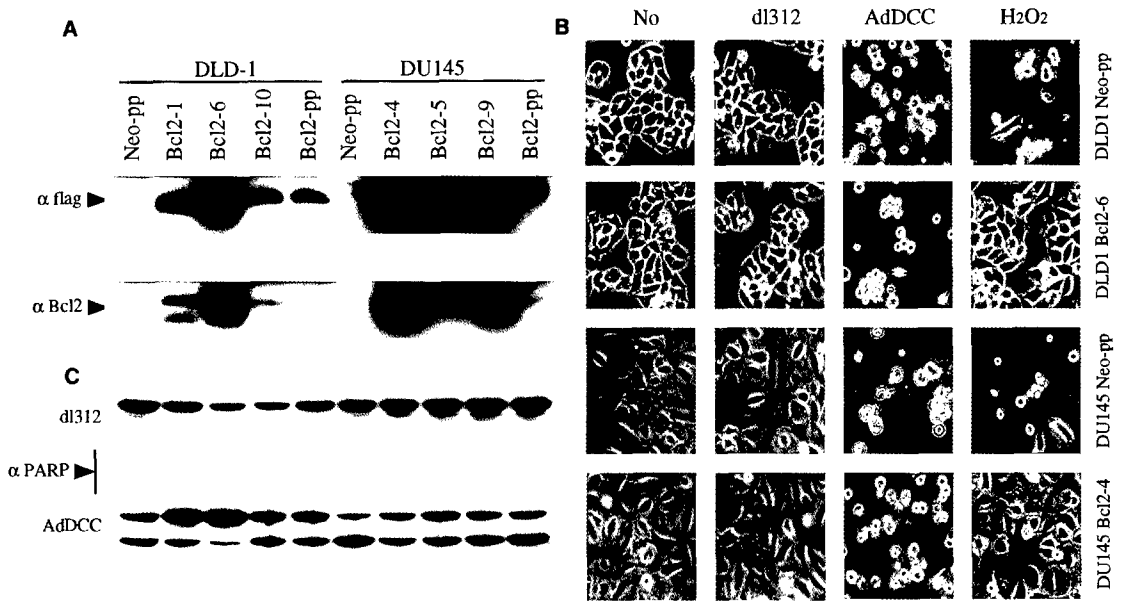


**Figure 4** Effect of Bcl-2 on DCC-induced apoptosis. DLD-1 and DU145 cells were transfected with pcDNA3 or pcDNA Bcl-2-flag vector and selected with G418. Pooled population as well as individual clones were obtained. Expression of the Bcl-2-flag protein was determined by Western blotting using anti-flag and anti-Bcl-2 antibodies (**a**). $Neo^R$ and Bcl-2 expressing DU145 and DLD-1 cells were mock infected, infected with d1312, AdDCC at MOI 5 or treated with 300 $\mu$M (DU145) or 500 $\mu$M (DLD-1) of $H_2O_2$. Cell morphology was monitored at 48 h post-treatment (**b**). Cells infected with d1312 and AdDCC were lysed and used for Western detection of PARP cleavage (**c**). No: mock treated. Neo-pp: pooled population of G418 resistant clones. Bcl-2-pp: pooled population of Bcl-2-transfected clones. Bcl-2-1: clone number 1 of Bcl-2-transfected cells

in DLD-1 cells (Figure 4a). Bcl-2 expression protected DU145 and DLD-1 cells from apoptosis induced by $H_2O_2$ (Figure 4b), indicating that the transfected Bcl-2 protein is functional. However, the functional Bcl-2 expression did not protect DU145 and DLD-1 cells from apoptosis induced by DCC (Figure 4b). PARP cleavage could be seen in the pooled Neo or Bcl-2 populations as well as individual Bcl-2 colonies of DU145 and DLD-1 cells (Figure 4c). Nevertheless, Bcl-2 expression reduced the ratio of the cleaved 85 kD to the intact 116 kD PARP (Figure 4c). These results suggest that the DCC-induced apoptosis process cannot be blocked, but may be slightly slowed down by Bcl-2.

To determine whether induction of apoptosis by DCC is a general phenomenon, we studied a total of twenty-two cell lines. These included three prostate carcinoma, one colon carcinoma, four breast carcinoma, three neuroblastoma, three osteosarcoma, one cervical carcinoma, five bladder carcinoma cell lines and one immortal breast epithelial and one immortal keratinocyte cell line. DCC expression induced either apoptosis or cell cycle arrest in all cell lines studied. The cells, based on their response to DCC, can be categorized into three groups: (a) rapid induction of apoptosis, (b) irreversible cell cycle arrest followed by apoptosis and (c) transient cell cycle arrest.

The DCC gene encodes a cell surface receptor of the Ig superfamily (Fearon *et al.*, 1990). Molecules of the Ig family can mediate cell-cell and cell-matrix interactions through a homotypic (i.e. receptor interacts with the same receptor) or a heterotypic fashion (i.e. receptor interact with a ligand or another receptor). So far, no homotypic interaction of DCC has been confirmed. However, it has been shown that DCC is a candidate component of the receptor for netrin-1 (Keino-Masu *et al.*, 1996; Chan *et al.*, 1996; Kolodziej *et al.*, 1996). Therefore, we also evaluated whether netrin-1 can stimulate or inhibit DCC-induced apoptosis. DU145 cells were infected with control, DCC adenovirus or mock infected in the absence or presence of netrin-1 protein. The kinetics of apoptosis was monitored by DNA fragmentation and microscopy. Our results indicate that exogenous netrin-1 protein has no effect on the DCC-induced apoptosis (data not shown). However, it was not clear whether all of DCC receptors were occupied by the netrin-1 protein under our experimental conditions.

Cell surface receptors usually transduce their signal through the cytoplasmic domain. To address the role of the cytoplasmic domain of DCC in inducing apoptosis, we generated recombinant adenovirus of a DCC mutant that lacks its cytoplasmic domain (Figure 5a). Successful expression of the mutant DCC was confirmed by Western (Figure 5b). Next, we determined the ability of this DCC mutant to induce apoptosis. We found that the wild-type DCC but not the mutant induced apoptosis, activated caspase-3 (Figure 5c and d). Our results demonstrate that the cytoplasmic domain is required for DCC to induce apoptosis, and confirm the involvement of caspase-3 in DCC-induced apoptosis.

As mentioned earlier, DCC expression did not induce apoptosis of PC-3 cells. Instead, DCC expression resulted in PC-3 cells with a flattened, enlarged morphology resembling G1 arrested cells. To determine whether DCC expression induces cell cycle arrest in some cell lines, human prostate carcinoma PC-3, osteosarcoma HOS and breast carcinoma MCF-7 cells were infected with the AdDCC adenovirus,

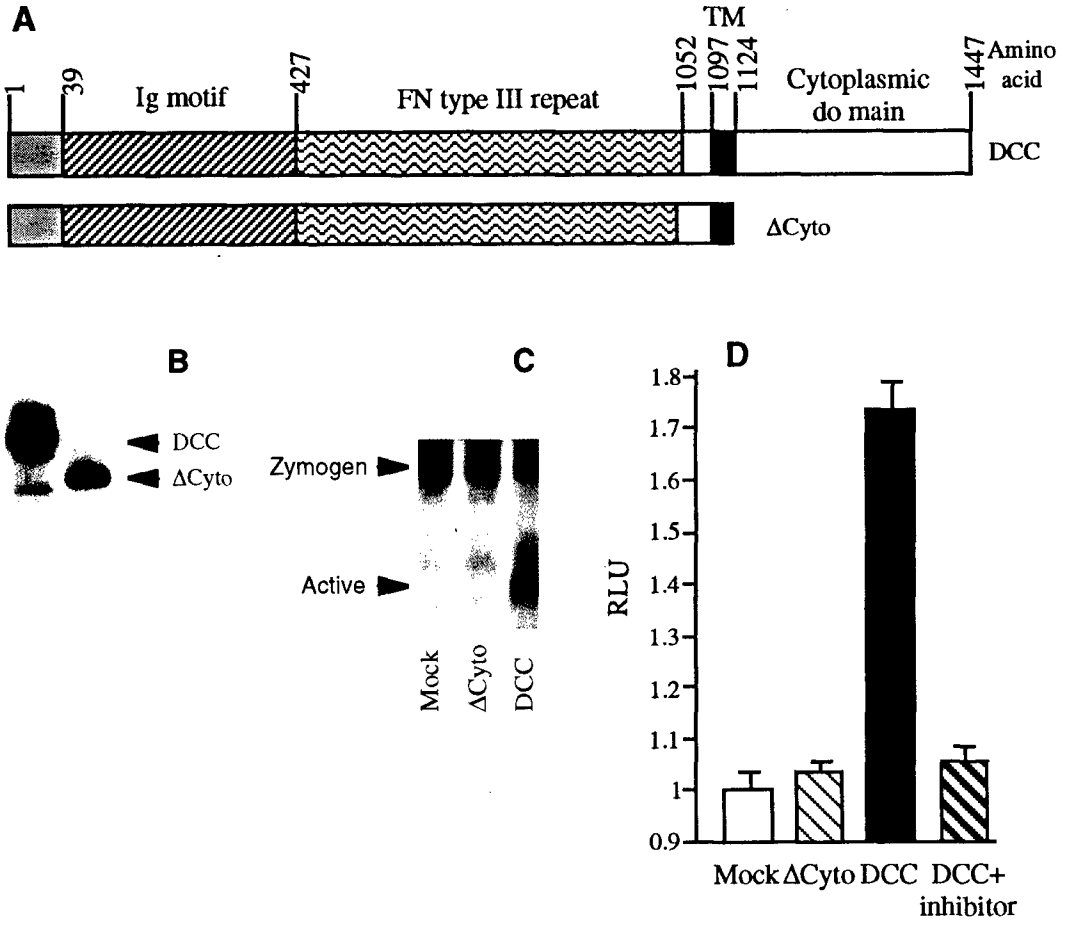


**Figure 5** Requirement of the DCC cytoplasmic domain in inducing apoptosis. (**a**) structure of the wild-type DCC and a mutant without the cytoplasmic domain. (**b**) DU145 cells were infected with AdDCC or AdΔcyto at MOI 5. Samples were collected at 24 h post-infection and used for western detection of DCC with an anti-DCC extracellular domain antibody. C: DU145 cells ($1 \times 10^6$) were infected with AdDCC, AdΔcyto at MOI 5 or mock infected. Samples were collected at 24 h post-infection and used for western detection of the active form of the caspase-3 protein. (**d**) samples from C were used for caspase-3 activity assay (bars) using a CPP32 colorimetric assay kit (Clontech). Caspase-3 specific inhibitor used was DEVD peptide. RLU stands for relative light unit. Bar represents standard deviations

AdCMV and dl312 control adenovirus or mock-infected. Samples were collected 72 h post-infection and used for cell cycle distribution analysis by flow cytometry. Mock and control adenovirus-infected cells had a similar G1, S and G2/M phase distribution, whereas DCC adenovirus-infected cells were arrested at the G2/M phase (Figure 6a). The G2/M population increased by more than twofold at 72 h post-infection of AdDCC in the asynchronized culture of PC-3, HOS and MCF-7 cells (Figure 6b) and cells were growth arrested (Figure 6c). Our data indicate that DCC expression induces a rapid G2/M cell cycle arrest in some cell lines.

Due to the nature of flow cytometry, we were uncertain whether DCC expression arrested cells at the G2 or the prophase, metaphase, anaphase or telophase of the cell division. To differentiate between these possibilities, PC-3 cells were infected with AdDCC, dl312 control adenovirus or mock-infected. Cells were fixed 72 h post-infection and used for immunocytochemistry analysis using an anti-DCC antibody. DCC expressing cells had a flatter morphology (Figure 7a) compared to the dl312 control- (Figure 7b) or mock-infected cells (data not shown). Cell nuclei were stained with DAPI. The nuclei of DCC expressing cells had an intact nuclear membrane and slightly condensed chromatin (Figure 7b and insert), a characteristic of early prophase cells. These results suggest that DCC expressing cells were arrested at the later G2/early prophase of mitosis. Three groups of molecules, i.e. cyclins, cyclin-dependent kinases and cyclin-dependent kinase inhibitors, have been identified and they are the key molecules in controlling cell cycle progression (Hunter and Pines, 1994). All the inhibitors identified so far are primarily involved in G1 control. Cdk4 and Cdk6, in association with cyclin D, are important in G1 progression. Cdk2, in association with cyclin E or cyclin A, is critical for G1 to S transition and S phase progression. Cdk1 (also known as Cdc2) in association with cyclin B is required for cell mitosis (Nurse, 1994; King *et al.*, 1994; Dunphy, 1994). The prophase cell cycle arrest by DCC may be due to an inhibition of Cdk1 activity. Therefore, Cdk1 kinase activity was compared in AdDCC-, control adenovirus- and mock-infected cells. Indeed, we found that DCC expression inhibited the Cdk1 kinase activity (Figure 7i).

DCC was suggested as a tumor suppressor. Recently, the mouse homologue of DCC has been

knocked out (Fazeli *et al*., 1997). Mice homozygous for the Dcc$^-$ died within 24 h after birth, and showed defects in axonal projections. No alterations in growth, differentiation, morphogenesis or tumorigenesis of intestine were observed in both homozygous and heterozygous mice as well as in Dcc$^{+/+}$<->Dcc$^{-/-}$ chimeras. These results fail to support a tumor suppressor function for DCC. However, the lack of

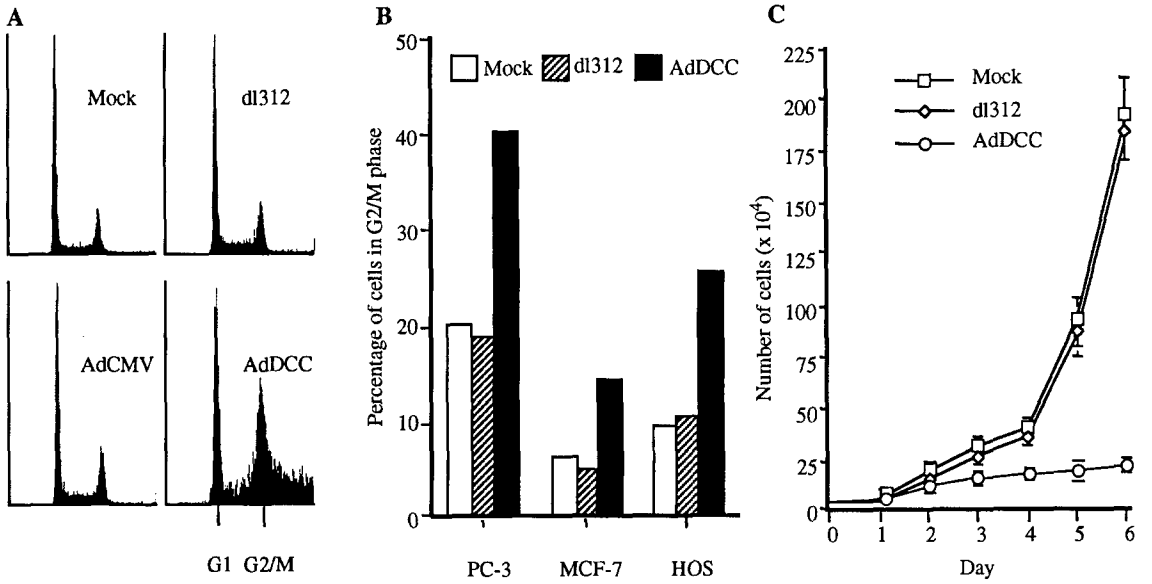


**Figure 6** DCC-induced G2/M cell cycle arrest. (**a**) PC-3 cells were infected with AdCMV or d1312 control adenovirus, AdDCC adenovirus or mock-infected. Cells were collected 72 h post-infection and used for flow cytometry analysis. (**b**) Percentage of the G2/M population in mock-, d1312 control- and AdDCC adenovirus-infected PC-3, MCF-7 and HOS cells. Samples were analysed at 72 h post-infection. C: PC-3 cells were infected with AdDCC, d1312 at MOI 5 or mock infected. Number of cells was counted daily for a period of 6 days. Three samples were used for each point and three independent experiments were performed. Bar represents standard deviations

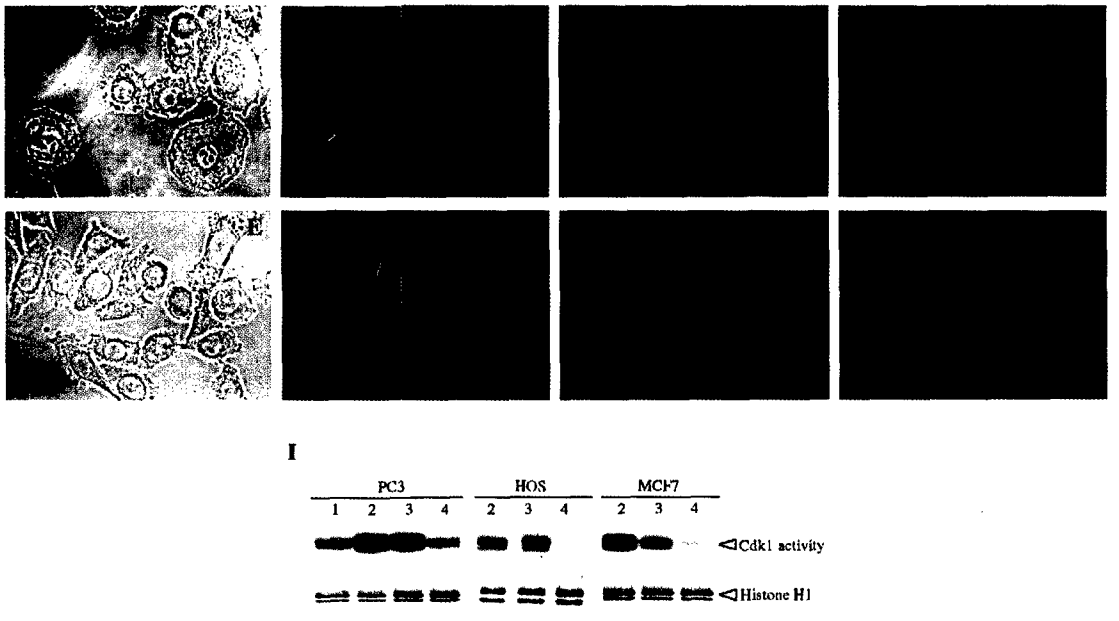


**Figure 7** Morphological features and Cdk1 activity of cells arrested by DCC. PC-3 cells were infected with d1312 or AdDCC adenovirus at multiplicity of infection 5. Cells were fixed at 72 h post-infection and used for immunocytochemistry. (**a**) phase contrast image of AdDCC-infected cells, (**b**) DAPI staining (blue color) of AdDCC-infected cells, (**c**) DCC staining (red color) of AdDCC-infected cells, (**d**) composite of DAPI and DCC staining of AdDCC-infected cells, (**e**) phase contrast image of d1312-infected cells, (**f**) DAPI staining (blue color) of d1312-infected cells, (**g**) DCC staining of d1312-infected cells, (**h**) composite of DAPI and DCC staining of d1312-infected cells, (**i**) PC3, HOS and MCF7 cells were mock-infected (2) or infected with d1312 control adenovirus (3) and AdDCC (4) at MOI 5. Samples were collected at 72 h post-infection and 500 $\mu$g of total protein were used for immunoprecipitation with anti-Cdk1 antibody, then for histone H1 phosphorylation assay. The upper panels show the phosphorylation of histone H1 by Cdk1, the lower panels show the loading of histone H1 protein. To show the specificity of kinase activity assay, 500 $\mu$g of mock-infected PC3 protein were immunoprecipitated with anti-Cdk1 antibody in the presence of 1 $\mu$g of Cdk1 immunogen peptide (1)

tumor in homozygous mice may be due to the early death of offspring. The result from heterozygous mice should be interpreted with caution. For example, heterozygous mice for BRCA1 or BRCA2 gene did not manifest defects or an increased rate of tumorigenesis (Liu *et al.*, 1996; Hakem *et al.*, 1996; Sharan *et al.*, 1997). In addition, the phenotype of a tumor suppressor gene loss in human may not be mimicked by the phenotype in mice. For instance, more than 50% of human colorectal tumors have a loss of function of the tumor suppressor gene p53. Yet, no colon tumor could be seen in p53 knockout mice (Donehower *et al.*, 1992). Therefore, it is still uncertain whether the DCC is a tumor suppressor gene. In fact, our data on induction of apoptosis and cell cycle arrest by DCC argue for a tumor suppressor function of DCC.

Interestingly, forced DCC expression induces programmed cell death and/or cell cycle arrest of tumor cells regardless of their p53 and Rb status, i.e. wild-type, mutant or null. The DCC protein is a cell surface receptor. So far two other cell surface receptors, Fas and TNFR1, have been shown to be capable of inducing apoptosis. The Fas/TNFR1 apoptotic signals are transduced by a cascade of molecular interactions involving TRADD, FADD and FLICE (Cleveland and Ihle, 1995; Fraser and Evan, 1996). These molecules interface through a highly homologous region termed death domain. However, no death domain or an obvious death domain homology can be identified in the cytoplasmic portion of the DCC protein. Therefore, it would be of great interest to define the pathway by which DCC induces apoptosis and cell cycle arrest.

## Materials and methods

### *Cells*

Human prostate carcinoma DU145, PC-3, LNCaP cells were cultured in RPMI 1640 with 5% FBS, colon carcinoma DLD-1 in DMEM with 5% FBS, breast carcinoma MCF7 cells in DMEM with 5% FBS, 10 $\mu$g/ml insulin, osteosarcoma HOS in RPMI 1640 with 10% FBS. Cell numbers were counted with the trypan blue exclusion method in a hematocytomer.

### *Western blotting*

Detection of DCC, $\beta$-actin, poly (ADP-ribose) polymerase, caspase-3, Bcl-2 and the flag epitope were performed as described previously (Chen *et al.*, 1995) with a monoclonal anti-DCC C-terminus antibody (PharMingen), a monoclonal anti-actin antibody (Sigma), the monoclonal C-2-10 anti-PARP antibody (Biomol), a monoclonal anti-caspase-3 (PharMingen), a monoclonal anti-Bcl-2 (DAKO) antibody and the M2 monoclonal anti-Flag antibody (Eastman Kodak).

### *Recombinant adenovirus*

Human DCC cDNA was cloned into the shuttle vector pAdE1CMV/pA. Recombinant DCC adenovirus was generated by a homologous recombination between pAdE1CMV/pA-DCC and the proviral vector pBHG10 in 293 cells. Once recombinant virus was formed, each viral clone was plaque-purified, amplified in 293 cells and characterized by genomic PCR (Kleinerman *et al.*, 1995). Three sets of primer were used separately. Primer set 1 (5′-CACTGAGGTGTCCACAGGAA-3′; 5′-GTTCAA-GGGAGGAGTCCAAC-3′) was used for identifying the presence of the DCC cDNA insert. Primer set 2 (5′-TCGTTTCTCAGCAGCTGTTG-3′; 5′-CATCTGAACTCA-AAGCGTGG-3′) was used for identifying the presence of viral sequences. Primer set 3 (5′-ATTACCGAAGAAATGG-CCGC-3′; 5′-CCCATTTAA-CACGCCATGCA-3′) was used for examining the presence of E1 region. An absence of the E1 gene indicates that recombinant adenovirus stock is free of wild-type adenovirus contamination. AdCMV adenovirus was generated by a homologous recombination between the shuttle vector without cDNA insert and the proviral vector. The human Ad5 mutant d1312 is a replication-defective adenovirus due to the deletion of the E1 gene (Jones and Shenk, 1979). The AdCMV and d1312 adenovirus were used as controls. To construct AdDCCΔcyto, a PCR fragment that extends from a portion of the FN type III repeat to amino acid 1124 was used to replace the DCC cytoplasmic domain. The pcDNA-DCCΔcyto plasmid was sequenced to avoid possible PCR mis-incorporation. The AdDCCΔcyto was constructed with procedures described above.

### *DNA fragmentation assay*

Cells were collected and lysed in 200 $\mu$l of cold lysis buffer (20 mM Tris pH 7.4 10 mM EDTA 0.2% Triton X100) on ice for 15 min with vigorous votexing every 5 min. The lysates were spun for 10 min in at 10 000 *g*. Supernatants were incubated with 50 $\mu$g/ml of DNase-free RNase A at 37°C for 1 h, then with 0.1 mg/ml of proteinase K, 0.1% SDS at 50°C overnight. Samples were extracted with phenol and chloroform. DNA was precipitated with ethanol, washed with 70% ethanol, dried and suspended in Tris-EDTA buffer. Samples were run on a 1.5% agarose gel.

### *Nuclear staining*

Cells were washed once in 1 $\mu$g/ml DAPI-methanol, stained in the DAPI-methanol solution at 37°C for 15 min, washed off the staining solution with methanol and mounted with anti-fade buffer.

### *Immunostaining*

Staining of DCC protein with peroxidase method was performed as described previously (Chen *et al.*, 1994). For immuno-fluorescent staining, cells were washed with PBS, fixed in 3.7% formaldehyde for 10 min and incubated with 0.2% BSA for 1 h to block non-specific binding. Samples were incubated with 20 $\mu$g/ml of anti-DCC antibody diluted in PBS-0.1% saponin for 1 h, washed with PBS, blocked with 5% normal donkey serum for 1 h and then incubated with 25 $\mu$g/ml donkey anti-mouse IgG conjugated with Texas red for 1 h. Samples were rinsed in PBS and mounted with anti-fade mounting buffer containing 75 ng/$\mu$l of DAPI. Cells were examined with Zeiss laser scan confocal microscope.

### *Flow cytometry*

Cell cycle distribution was analysed by flow cytometry as described previously (Chen *et al.*, 1995).

### *Addition of netrin-1*

Cells were mock-infected, or infected with dl312 or AdDCC at a MOI of five in the presence of 0, 0.3 and 3 $\mu$g/ml of netrin-1 protein, respectively.

### *Bcl-2 expression*

DU145 and DLD-1 cells were transfected with pcDNA3 or pcDNA Bcl-2-flag vector using lipofection method (25), and

selected with 400 μg/ml and 700 μg/ml of G418, respectively. Pooled population (>100 colonies) of Neo and Bcl-2 as well as individual colonies of Neo and Bcl-2 were obtained. Successful expression of the Bcl-2 protein was confirmed by Western blotting using anti-flag and anti-Bcl-2 antibodies. Functional expression of the Bcl-2 protein was determined by a protective effect of Bcl-2 on apoptosis induced by $H_2O_2$.

*Cdk1 kinase assay*

The activity of Cdk1 was measured by immunoprecipitation of Cdk1 with a polyclonal anti-Cdk1 antibody (Santa Cruz Biotechnology) following by H1 histone phosphorylation assay as described previously (Cipriano and Chen, 1998).

*Sequence homology analysis*

Possible sequence homology between the DCC cytoplasmic region and the death domain from Reaper, Fas, TNFR1, TRADD, FADD and RIP was analysed with the LaserGene software (DNASTAR, Inc.) on a Macintosh computer.

**Abbreviations**

Cdk, cyclin-dependent kinase; DAPI, 4′, 6-Diamidine-2′-Phenylindole Dihydrochloride; DMEM, Dulbecco modified minimum essential medium; EGF, epidermal growth factor; Ig, immunoglobulin; MOI, multiplicity of infection; FBS, fetal bovine serum; PARP, poly (ADP-ribose) polymerase.

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human ovarain cancer cells. Co-cultures were grown up for 24h under standard tissue culture conditions. PEO1 cells were then removed from the chamber inserts by gentle trypsinisation, plated into 96-well plates and subjected to chemosensitivity testing. Cells were treated with doxorubicin and cisplatin and then an MTT assay was carried out 72h later. Control PEO1 cells were grown up in chamber inserts in the presence of non-activated MM6 cells. An apparent reduction in drug sensitivity in PEO1 ovarain cancer cells following co-culture with MM6 cells was noted. Data shown are the mean for at least 3 assays IC50 values: Cisplatin= 0.35uM control, 0.79uM following co-culture ($p = 0.02$); doxorubicin= 28.8uM control, 53.7uM following co-culture ($p = 0.05$). In order to understand the possible mechanisms underlying our observations, we are looking at changes in the apoptotic response of PEO1 cells under the co-culture conditions. In particular, IL-6 serum level has been reported to be an important prognostic indicator in ovarian cancer. Our ongoing studies are therefore, focussing on IL-6 as this is a cytokine closely associated with TAMs. Bcl2 levels will be looked at in addition to parameters used to measure apoptotic responses.

**#1323 A Novel Role of Tissue Factor Pathway Inhibitor-2 (TFPI-2) in Apoptosis of Malignant Human Gliomas.** Anastasia V. Tasiou, Santhi D. Konduri, Niranjan Yanamandra, and Jasti S. Rao. *University of Texas M. D. Anderson Cancer Center, Houston, TX.*

Tissue factor pathway inhibitor-2 (TFPI-2) is a 32 kDa serine protease inhibitor abundant in the extracellular matrix. Earlier studies in our laboratory showed that the expression of TFPI-2 is lost during tumor progression and that it also played a role in tumor invasiveness in vitro and in vivo in human gliomas. Collectively, these results suggested a way to determine whether TFPI-2 is necessary for cell survival and inhibition of tumor formation in nude mice. In the present paper we wanted to investigate whether overexpression of TFPI-2 inhibited cell growth and induced apoptosis. We found that p-ERK levels decreased in TFPI-2 overexpressing clones (SNB19) and increased in TFPI-2 downregulated clones (Hs683). We also looked at levels of Bax/Bcl-2, caspases (nos. 9, 8, 7, 3), PARP, cytochrome-c, and Apaf-1. We found that Bax protein levels increased in overexpressing TFPI-2 clones (SNB19) and decreased in clones carrying the TFPI-2 construct in antisense orientation (Hs683). Caspase (9,7,3), cytochrome-c, Apaf-1, and PARP levels were increased in SNB19 cells and decreased in Hs683 cells. Activity assay of caspase 9 and 3 revealed higher activity in overexpressing clones (SNB19) but lesser activity in downregulated clones (Hs683) compared to controls. To our knowledge, this is the first report that TFPI-2 plays a novel role in cell survival in human gliomas.

**#1324 Identification of a DCC Interaction Protein (DIP13) and Its Potential Role in DCC Signaling.** Jiayou Liu, Fayi Yao, Russell L. Finley, and Yong Q. Chen. *Center for Molecular Medicine and Genetics, Wayne State University, Detroit, MI, and Dept. of Pathology, Wayne State University, Detroit, MI.*

Deleted in Colorectal Cancer (DCC) is one of the genes frequently altered in many human tumor types and has been suggested to be a tumor-suppressor gene. Recently, our group and others have demonstrated that forced expression of DCC induces apoptosis or cell cycle arrest (Chen et al., 1999; Mehlen et al., 1998). DCC encodes a cell surface receptor. Its cytoplasmic domain is required for the induction of apoptosis (Chen et al., 1999). We are interested in understanding how DCC apoptotic signals are transduced. As a first step, we set out to identify proteins that interact with the cytoplasmic domain of DCC. Here we report the identification of one of these proteins, DIP13 (for DCC Interacting Protein 13). We cloned the full-length cDNA and determined the exon-intron junctions of the DIP13 gene. We confirmed the interaction of DIP13 with DCC by co-immunoprecipitation and western blotting. We mapped the DIP13-interaction site to a region of 35 amino acids in the center of the DCC cytoplasmic domain, a region required for DCC to induce apoptosis. We also mapped the DCC-interaction site on DIP13 to a region of 192 amino acids that comprises a PTB domain. Interestingly, there is evidence that DIP13 interacts with AKT2 and, to a lesser degree, with AKT1 and AKT3. AKT proteins are a family of serine/threonine kinases that play a central role in growth responses to insulin and various other stimuli and in suppressing apoptosis. All together, we propose that DIP13 mediates the DCC apoptotic signal by interfering the function of AKT. Chen et al., 1999, Oncogene 18, 2747-2754. Mehlen et al., 1998, Nature 395, 801-804.

**#1325 Downregulation of Integrin $\alpha v\beta 3$ Expression and Integrin Mediated Signaling in Glioma Cells by Adenovirus Mediated Transfer of Antisense uPAR and Sense p16 Gene.** Yoshiaki Adachi, Sajani S. Lakka, Nirmala Chandrasekar, Sanjeeva Mohanam, Yoshiaki Kin, and Jasti S. Rao. *University of Texas M. D. Anderson Cancer Center, Houston, TX.*

The interaction between the extracellular matrix (ECM) and its cell surface receptor integrins leads not only to cell adhesion but also to intracellular signaling events that affect cell migration, proliferation, and survival. The vitronectin (VN) receptor, $\alpha v\beta 3$ integrin, is of key importance in glioma cell biology. Recently it was revealed that urokinase-type plasminogen activator receptor (uPAR) expression is coregulated with $\alpha v\beta 3$ integrin-mediated cell spreading on VN. Thus we expected that adenovirus-mediated downregulation of uPAR and overexpression of p16 would downregulate $\alpha v\beta 3$ integrin expression and integrin-mediated signaling in glioma cells. To test this hypothesis we used replication- deficient adenovirus vector containing antisense uPAR expression cassettes, an adenovirus vector containing wild-type p16 cDNA, and a bicistronic adenovirus construct contains antisense uPAR and p16 sense expression cassettes in the deleted E1 region. SNB19 malignant glioma cell lines infected with the construct and treated with VN showed decreased $\alpha v\beta 3$ integrin expression and integrin-mediated cell biological effects, including adhesion, migration, proliferation and survival.

**#1326 E-Cadherin and Plakoglobin-Mediated Regulation of Apoptosis in Squamous Carcinoma Cell Lines.** Manijeh Pasdar, Annissa Wong, Laifi Li, and Anita Gilchrist. *University of Alberta, Edmonton, AB, Canada.*

Programmed cell death or apoptosis is a fundamental cellular process which occurs during development and in the maintenance of cell number, tissue homeostasis and defence against pathological agents. In epithelial cells, apoptosis regulates growth and morphogenesis and is itself, regulated by both cell-cell and cell-matrix adhesion. Cell-cell adhesion in epithelia is regulated by the adhesion receptors cadherins and the cadherin-associated proteins $\alpha$-catenin, $\beta$-catenin and $\gamma$-catenin/ plakoglobin (Pg). We previously showed (Hakimelahi et.al. JBC, 257:10905-10911, 2000) that high levels of plakoglobin expression in E-cadherin and Pg-deficient human SCC9 cells leads to uncontrolled growth and foci formation. Concurrent with the change in growth characteristics we observed a pronounced inhibition of apoptosis. This correlated with an induction of expression of *Bcl-2*, a prototypic member of apoptosis-regulating proteins, and decreased activation of caspase-3, an executor of programmed cell death. Here, we show that introduction of E-cadherin into the Pg-expressing cells leads to the induction of contact inhibition of growth associated with apoptosis. This growth regulatory effect of the E-cadherin expression coincided with the disappearance of *Bcl-2* protein without a significant change in the levels of the mRNA for this protein. Our data suggest that the role of E-cadherin in growth regulation may be mediated via its inhibitory effect on the stability of *Bcl-2* protein.

**#1327 Characterization and Functional Analysis of an E-Cadherin Cytosolic Proteolytic Domain.** Jonathan Rios-Doria, Kathleen C. Day, Xin Zhao, and Mark L. Day. *Comprehensive Cancer Center, University of Michigan, Ann Arbor, MI.*

The balance between cell survival and cell death is a critical one required for tissue homeostasis. In the prostate and mammary gland, alteration of hormone levels results in cell death and tissue involution. Potential effectors of hormone action include the epithelial adhesion molecule E-cadherin. Our lab was the first to show that inhibition of E-cadherin-dependent aggregation resulted in cell death following apoptotic stimuli. Further experiments demonstrated that this loss of cell-cell adhesion was closely associated with proteolytic cleavage in the cytoplasmic domain of the mature 120kDa form of E-cadherin. We have shown that this cleavage removes the $\beta$-catenin binding domain, rendering E-cadherin functionless. We are investigating the mechanism of E-cadherin proteolysis, and whether the loss of E-cadherin function is involved with the regulation of apoptosis. Preliminary data indicate that the cysteine protease, calpain, is involved in the proteolysis of E-cadherin. Examination the E-cadherin amino acid sequence reveals putative calpain recognition sites. We demonstrate that cell-permeable calpain inhibitor I can effectively inhibit E-cadherin proteolysis. And addition of calpain to immunopurified E-cadherin can reconstitute proteolysis *in vitro*. E-cadherin mutants have been generated at the proposed proteolytic sites, and they have been cloned into the PCDNA3 vector under the CMV promoter. We are able to express the mutant E-cadherin cDNA in prostate and epithelial cell lines. We have developed transient assays to study E-cadherin proteolysis in these cell lines, and we are examining whether these mutants can inhibit proteolysis in the cellular environment. We believe these results will elucidate the mechanism of E-cadherin cleavage, and delineate the role of proteolysis in cell adhesion and cell survival. These results could further support our hypothesis that E-cadherin-mediated adhesion provides a survival signal to tumor cells.

## MOLECULAR BIOLOGY 10: Telomeres, Chromatin, and Senescence

**#1328 A Quantitative Assay of Telomerase Activity.** Yuebo Gan, Jie Lu, Andy Johnson, M. Guillaume Wientjes, and Jessie LS Au. *The Ohio State University, Columbus, OH.*

Telomerase is a ribonucleoprotein that extends telomeres at the ends of chromosome. It is generally accepted that the currently available assay for telomerase, i.e., telomeric repeat amplification protocol (TRAP) lacks quantitative precision, in part due to the presence of inhibitors in the protein extracts that inhibit PCR amplification. The present study was to develop a quantitative telomerase assay with greater precision and sensitivity. Several human cancer cell lines as well as patient tumors were used in the analysis. This new method used the primer extension method as in TRAP, plus the following modifications: (a) used a lysis buffer that yielded complete lysis of nuclei; (b) removal of PCR inhibitors by phenol/chloroform extraction after primer extension; and (c) used primers for the internal standard that were designed to reduce their competition with the telomerase products for PCR. The modified method showed a good correlation ($r^2=0.99$, $p<0.001$) between telomerase amount (expressed as total protein in cell

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